Published in final edited form as:

DNA Repair (Amst). 2014 October ; 22: 41–52. doi:10.1016/j.dnarep.2014.07.008.

N-terminal domains of human DNA polymerase lambda promote primer realignment during translesion DNA synthesis

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Abstract

The X-family DNA polymerases λ (Pol λ) and β (Pol β) possess similar 5'-2-deoxyribose-5-phosphatylase (dRPase) and polymerase domains. Besides these domains, Pol λ also possesses a BRCA1 C-terminal (BRCT) domain and a proline-rich domain at its N terminus. However, it is unclear how these non-enzymatic domains contribute to the unique biological functions of Pol λ . Here, we used primer extension assays and a newly developed high-throughput short oligonucleotide sequencing assay (HT-SOSA) to compare the efficiency of lesion bypass and fidelity of human Pol β , Pol λ and two N-terminal deletion constructs of Pol λ during the bypass of either an abasic site or a 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) lesion. We demonstrate that the BRCT domain of Pol λ enhances the efficiency of abasic site bypass by approximately 1.6-fold. In contrast, deletion of the N-terminal domains of Pol λ did not affect the efficiency of 8-oxodG bypass relative to nucleotide incorporations opposite undamaged dG. HT-SOSA analysis demonstrated that Pol λ and Pol β preferentially generated –1 or –2 frameshift mutations when bypassing an abasic site and the single or double base deletion frequency was highly sequence dependent. Interestingly, the BRCT and proline-rich domains of Pol λ cooperatively promoted the generation of –2 frameshift mutations when the abasic site was situated within a sequence context that was susceptible to homology-driven primer realignment. Furthermore, both N-terminal domains of Pol λ increased the generation of –1 frameshift mutations during 8-oxodG bypass and influenced the frequency of substitution mutations produced by Pol λ opposite the 8-oxodG lesion. Overall, our data support a model wherein the BRCT and proline-rich domains of Pol λ act cooperatively to promote primer/template realignment between DNA strands of limited sequence homology. This function of the N-terminal domains may facilitate the role of Pol λ as a gap-filling polymerase within the non-homologous end joining pathway.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Keywords

X-family DNA polymerases; Abasic site; Translesion DNA synthesis; Lesion bypass; Mutagenic analysis

1. Introduction

Oxidative DNA damage is proposed to be a major contributor to carcinogenesis and aging [1,2]. Two of the most common forms of oxidative DNA damage generated within human cells are apurinic/apyrimidinic (AP) sites and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) lesions. It is estimated that approximately 10,000 spontaneous AP sites [3,4] and 1000-10,000 8-oxodG sites [5] are generated in each mammalian cell every day. These lesions are highly mutagenic, as AP sites have lost all coding potential, and 8-oxodG possesses dual coding potential due to its ability to adopt either an *anti* or *syn* conformation within a polymerase active site [6]. While in the *anti* conformation, a templating 8-oxodG correctly base pairs with an incoming dCTP by forming a Watson-Crick base pair. However, while in the *syn* conformation, a templating 8-oxodG lesion utilizes its Hoogsteen edge to preferentially form an incorrect base pair with an incoming dATP [7]. Under normal conditions, AP sites and 8-oxodG lesions are removed through the base excision repair (BER) pathway, and the resulting gap is filled by the action of a repair DNA polymerase such as polymerase λ (Pol λ) or β (Pol β). However, under conditions of significant oxidative stress, both strands of genomic DNA may become damaged, and therefore Pol λ or Pol β may be required to bypass damaged sites, a process termed translesion DNA synthesis (TLS).

Pol λ and Pol β are members of the X-family, one of six families of DNA polymerases (A, B, C, D, X, and Y). Although both Pol λ and Pol β are template-directed DNA polymerases, possess 5'-2-deoxyribose-5-phosphate lyase (dRPase) activity, lack 3'→5' exonuclease activity, and exhibit low processivity when replicating non-gapped DNA substrates, these enzymes have distinct biological roles *in vivo* (reviewed in reference [8]). For instance, Pol β plays a critical role in short-patch base excision repair (BER) [9,10] and may catalyze strand-displacement synthesis during PCNA-independent long-patch BER [11–13]. In contrast, Pol λ has been proposed to act as a gap-filling polymerase during repair of double-stranded DNA breaks within the nonhomologous end joining (NHEJ) pathway [14–16], although Pol λ also appears to possess a secondary role during BER of oxidative lesions [17,18].

Human Pol λ and Pol β both possess conserved dRPase and polymerase domains (Fig. 1), and share 32% amino acid sequence identity (54% sequence homology) within these domains [19,20]. However, unlike Pol β , Pol λ additionally contains a purported nuclear localization signal (NLS), a breast cancer susceptibility gene 1 C-terminal (BRCT) domain and a proline-rich domain at the N terminus (Fig. 1). BRCT domains are known to mediate protein-DNA and protein-protein interactions, and this domain is required for Pol λ to function within reconstituted NHEJ systems [14,21]. The proline-rich domain has been shown to limit Pol λ strand-displacement synthesis [15], and influence the rate and fidelity of Pol λ nucleotide incorporation into undamaged DNA substrates [22,23]. The proline-rich

domain is also phosphorylated by the cyclin-dependent kinase Cdk2/cyclin A complex [24], an event which may regulate the *in vivo* functions of Pol λ . Although the N-terminal domains of Pol λ likely contribute to the unique biological activities of this enzyme, the exact roles of these domains are unclear.

To investigate the potential functions of the N-terminal domains of Pol λ , we sought to determine how the N-terminal domains influence the efficiency of lesion bypass and the fidelity of Pol λ nucleotide incorporation during TLS of AP and 8-oxodG sites. To quantify the types and frequencies of errors induced by oxidative lesion bypass, we utilized a high-throughput short oligonucleotide sequencing assay (HT-SOSA) that was recently developed in our laboratory [25]. Our data support a model wherein the N-terminal domains of Pol λ increase the frameshift error rate of Pol λ opposite damaged sites by promoting primer/template realignment. This function of the N-terminal domains of Pol λ may enable Pol λ to catalyze the extension of primer/template junctions containing damaged bases or limited sequence homology, such as those encountered during the gap filling step of NHEJ.

2. Materials and methods

2.1. Materials

Reagents were purchased from the following companies: T4 DNA ligase from New England Biolabs, OptiKinase from USB Corporation, [γ ³²P]ATP from Perkin Elmer, ATP from Thermo Scientific, and dNTPs from GE Healthcare. Full-length human fPol λ (1-575), dPol λ (132-575), tPol λ (245-575) and full-length human Pol β (1-335) were produced and purified as previously described [22,26,27].

2.2. DNA substrates

The synthetic 40-mer DNA template containing 8-oxodG (40-mer-8oxodG) was purchased from Midland Certified Reagent Company (Table 1). All other synthetic DNA oligomers were purchased from Integrated DNA Technologies (Table 1 and Supplementary Table SI). The stable AP site analog tetrahydrofuran was used in place of a natural AP site to improve the stability of damaged DNA templates. All DNA oligomers were gel purified by using denaturing polyacrylamide gel electrophoresis (PAGE, 17% polyacrylamide, 8M urea). Upstream primers were 5'-[³²P]-radiolabeled by incubating the primer of interest with OptiKinase and [γ ³²P]ATP for 3 h at 37 °C. Gapped DNA substrates were generated by annealing the indicated 5'-[³²P]-radiolabeled upstream primer and a 5'-phosphorylated downstream primer with the corresponding template in a 1.00:1.25:1.15 molar ratio, respectively. The primer-template DNA substrates were similarly prepared by annealing a 5'-[³²P]-radiolabeled upstream primer with the appropriate template at a 1.00:1.15 molar ratio, respectively. DNA substrate annealing solutions were heat denatured by incubation at 75 °C for 5 min, followed by slow cooling to 25 °C over several hours.

2.3. Reaction buffer

All kinetic and HT-SOSA reactions were performed in optimized reaction buffer L (50 mM Tris, pH 8.4 at 37 °C, 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 5 mM DTT, 10% glycerol and 0.1 mg/mL BSA) for fPol λ , dPol λ and tPol λ or reaction buffer B (50 mM Tris,

pH 7.8 at 37 °C, 50 mM NaCl, 5mM MgCl₂, 0.1 mM EDTA, 5mM DTP, 10% glycerol and 0.1 mg/mL BSA) for Polβ. All reported concentrations are final after mixing and all reactions were performed at 37 °C.

2.4. Running start assays

A pre-incubated solution containing 100 nM of a polymerase and 100 nM of a 5'-[³²P]-labeled DNA substrate was rapidly mixed with a solution containing 200 μM of each of the four dNTPs. The reaction mixtures were quenched at various times by the addition of EDTA to a final concentration of 0.37 M. A rapid chemical quench flow apparatus (KinTek) was used for kinetic experiments with reaction times ranging from milliseconds to several minutes. Reaction products were resolved by using denaturing PAGE, and quantified by using a Typhoon Trio (GE Healthcare).

Analysis of the running start assays was performed as previously described [28,29] by calculating the relative lesion bypass efficiencies (lesion bypass%) of each polymerase as a function of reaction time. For each time point *t*, lesion bypass% was calculated as the ratio of the bypass events to the encounter events (Eq. (1)):

$$\text{lesion bypass\%} = \frac{B}{E} \times 100\% = \frac{B}{[21 - \text{mer}] + B} \times 100\% \quad (1)$$

where the total lesion bypass events (B) was the sum of the concentrations of all products with sizes greater than or equal to the 22-mer, and the total lesion “encounter” events (E) was the summation of the 21-mer product concentration and the total lesion bypass events (B). To semi-quantitatively define the lesion bypass efficiency, the t_{50}^{bypass} was defined as the time required to bypass 50% of the total lesions encountered by each polymerase.

2.5. High-throughput short oligonucleotide sequencing assay

HT-SOSA was performed as previously described [25] with the following modifications. A pre-incubated solution containing 100 nM of a polymerase and 100 nM of a 5'-[³²P]-labeled, gapped DNA substrate (Supplementary Table S2) was mixed with a solution containing 200 μM of each of the four dNTPs. The optimal time for extension for each extension reaction was determined empirically. Extension reactions with 7-nucleotide gapped DNA substrates that were undamaged, contained an AP site or contained an 8-oxodG site were incubated for 1 min, 10 min, or 1 min, respectively. Extension reactions with single-nucleotide gapped DNA substrates that were undamaged, contained an AP site or contained an 8-oxodG site were incubated for 15 s, 1 h or 15 s, respectively. After extension, T4 DNA ligase (4U/μL) and ATP (0.5 mM) were added to the reactions. The reactions were then incubated for an additional 4 h at 37 °C to allow for ligation of the newly replicated strand to the 5'-phosphorylated downstream primer. After generation of the lesion bypass products, the full-length, newly synthesized DNA strands were effectively separated from the template strands by using denaturing PAGE, as the 13-mer and 16-mer primers annealed 5 bases downstream from the 3'-end of the template strands (Scheme 1 and Supplementary Table S2).

Sequencing libraries were generated by PCR amplification of each purified HT-SOSA bypass product with a forward primer containing a four nucleotide barcode sequence and a

reverse HT-SOSA primer (Supplementary Table SI). The PCR products were gel purified by using the QIAquick Gel Extraction Kit (Qiagen) and the remaining adapter sequences necessary for next-generation sequencing were added through a second round of PCR with Illumina PCR primers 1 and 2 (Supplementary Table SI). The PCR products were then gel purified as described above and the DNA products were then mixed in equal molar ratios. The combined sequencing library solution was then subsequently mixed with an equal molar amount of a sequence library derived from the genome of bacteriophage Φ X to increase sequence diversity. The final sequencing library solution was subjected to next-generation sequencing by using a HiSeq 2000 DNA sequencer (Illumina). This method is summarized in Scheme 1.

2.6. Analysis of DNA sequences

DNA sequencing reads were sorted and analyzed as previously described [25]. Briefly, the raw sequence reads that matched the Φ X genome and all sequence reads that contained one or more base calls which were not identified with greater than 99% accuracy (Phred quality score of less than 20) were removed. The remaining sequence reads were sorted into groups corresponding to the barcode sequences. After sorting, erroneous sequences were further removed by eliminating all sequence reads that did not perfectly match the reference sequences within the first six nucleotides, which consist of the four nucleotide barcode sequence and two adjacent nucleotides. Each batch of sequence reads (Supplementary Table S3) was then analyzed by using the “The Next-Generation Sequencing Position Counter” software [25] to align the sequences to a defined reference sequence and to quantify the total number of matches, mismatches (*i.e.* substitutions), insertions, and deletions generated by the X-family polymerases at each template position.

3. Results

3.1. The Pol λ BRCT domain increases the efficiency of AP site bypass

Pol λ [30–32] and Pol β [32,33] bypass AP sites *in vitro*, and full-length Pol λ has been shown to bypass AP sites more efficiently than Pol β when tested under similar conditions [32]. Interestingly, we have demonstrated that the N-terminal domains of human Pol λ alter the efficiency and fidelity of nucleotide incorporation into undamaged DNA substrates by using pre-steady-state kinetic analysis [22,23]. However, it is not clear how these non-enzymatic domains contribute to the polymerase activity of Pol λ during TLS. We therefore sought to investigate the influence of the BRCT and proline-rich domains of Pol λ during AP lesion bypass. To this end, we compared the AP bypass abilities of full-length Pol λ (fPol λ), a deletion construct of Pol λ (dPol λ) which lacks the BRCT domain, truncated Pol λ (tPol λ) which lacks the entire N-terminal region, and full-length Pol β which possesses a domain structure similar to tPol λ (Fig. 1). We previously determined that the nucleotide incorporation efficiencies of fPol λ , dPol λ , and tPol λ are higher when replicating DNA substrates containing a gap size of up to 7 nucleotides when compared to non-gapped DNA substrates [23]. Therefore, we chose to initially evaluate AP bypass by using control (18-16/41-mer-ctl) and damaged (18-16/41-mer-AP-5′G) DNA substrates containing a 7-nucleotide gap (Table 1). When replicating the undamaged 18-16/41-mer-ctl substrate, fPol λ , dPol λ , and tPol λ paused at template position 25, immediately upstream from the 16-

mer primer (Fig. 2A, C and E), indicating that upstream primer elongation by fPol λ , dPol λ and tPol λ was blocked by the downstream 16-mer primer. In contrast, Pol β paused at template position 25 only slightly before synthesizing the full 41-mer product (Fig. 2G), thus confirming the superior strand-displacement activity of Pol β [20]. When replicating the damaged 18-16/41-mer-AP-5'G substrate containing a site-specifically placed AP site, strong pausing by each polymerase was observed at a template position immediately preceding the lesion (Fig. 2B, D, F and H), indicating that all four enzymes were inhibited for nucleotide incorporation opposite the AP site. Additionally, AP site bypass catalyzed by fPol λ , dPol λ and tPol λ resulted in the accumulation of a 24-mer product (Fig. 2B, D and F), suggesting that AP site bypass catalyzed by these polymerases predominantly led to the generation of -1 frameshift mutations. To qualitatively define the relative lesion bypass efficiency of each enzyme by using our previously established methods [28,29], we calculated the time required for each polymerase to bypass 50% of the AP sites encountered (t_{50}^{bypass}) relative to replication of 50% of the corresponding dA sites (t_{50}) within the undamaged template (Table 2). This semi-quantitative analysis revealed that fPol λ bypassed the AP site approximately 1.6-fold more efficiently than dPol λ , tPol λ or Pol β , even though fPol λ traversed the corresponding dA site within the undamaged DNA substrate approximately 4.3-fold less efficiently than either dPol λ or tPol λ , and 9.3-fold less efficiently than Pol β . Therefore, when replicating the gapped DNA substrates, the BRCT domain of fPol λ increased the rate of AP site bypass, but appeared to reduce the overall nucleotide incorporation rate of fPol λ opposite undamaged DNA nucleotides. We concluded that the BRCT domain of fPol λ enhances the efficiency of AP lesion bypass.

An interaction between the 5' phosphate of a downstream primer within a gapped DNA substrate and the dRPase domain of either fPol λ or Pol β stimulates polymerase activity [20,34,35]. To determine if the N-terminal domains of Pol λ influence AP bypass in the absence of a downstream primer, we examined the abilities of fPol λ , dPol λ , tPol λ and Pol β to extend both a non-gapped control (18/41-mer-ctl) and a damaged (18/41-mer-AP-5'G) DNA substrate (Table 1). Interestingly, fPol λ , dPol λ and tPol λ failed to bypass 50% of the AP sites encountered within the non-gapped 18/41-mer-AP-5'G damaged substrate within 3 h (Table 2 and Supplementary Fig. S1), whereas the efficiency of Pol β -catalyzed AP site bypass was similar between the gapped and non-gapped, damaged DNA substrates. Thus, an interaction between Pol λ and the downstream primer within a gapped DNA substrate is critical for Pol λ -catalyzed AP site bypass, whereas this interaction is dispensable for Pol β -catalyzed AP bypass. Considering that Pol β and tPol λ possess similar domain structures, this difference in the requirement for a downstream primer suggests that Pol β and Pol λ utilize distinct molecular mechanisms for AP site bypass.

3.2. The N-terminal domains of Pol λ act to promote a homology-driven strand slippage mechanism of AP site bypass

To investigate if the N-terminal domains of human Pol λ influence the type or frequency of mutations induced by TLS of an AP site, we used our newly developed HT-SOSA approach [25]. To this end, we generated DNA substrates that possessed a 7-nucleotide gap, and were either undamaged or contained a site-specifically placed AP site (Supplementary Table S2). The upstream primer of these HT-SOSA DNA substrates annealed five nucleotides

downstream of the 3' end of the template strand to facilitate isolation of the lesion bypass products from the template strand by using denaturing PAGE (Scheme 1). In order to determine if the preferred action of a polymerase opposite the AP site is sequence dependent, five damaged substrates containing a 7-nucleotide gap were created, each bearing different templating bases immediately 5' to the AP site. Importantly, the damaged DNA substrate 13-16/41-mer-AP-5'T contained a template dT both immediately 3' and 5' to the AP site in order to test the possibility that a polymerase is capable of bypassing an AP site through a homology-driven strand slippage mechanism (see Section 4). Additionally, to control for the prospect that a repeated base 5' to the AP site influences polymerase fidelity, a damaged template bearing two template dCs immediately 5' to the AP site was also created. These control and damaged DNA substrates were then extended by the action of fPol λ , dPol λ , tPol λ , or Pol β , and the newly replicated strands were subsequently isolated, amplified and sequenced as described in Scheme 1. By using HT-SOSA, an average of 2.6 million nucleotide sequences generated from each combination of polymerase and DNA substrate were analyzed to provide statistically robust data sets (Supplementary Table S3). As an internal control for errors introduced by the HT-SOSA approach, we determined the average error frequencies within a control flanking region (positions -14 to -4 relative to the lesion site) of each sequence, which was initially derived from the upstream primer and thus, was not replicated by fPol λ , dPol λ , tPol λ or Pol β (Scheme 1 and Supplementary Table S2). The average base substitution, insertion and deletion frequencies within this control region were calculated to be 0.14%, 0.016% and 0.077%, respectively. Thus, the overall background error frequency is calculated to be 0.23% at each template position. Importantly, this background error frequency was found to be at least 110-fold below the error frequency of each polymerase opposite each lesion site tested (Supplementary Tables S4-S7).

HT-SOSA analysis revealed that fPol λ , dPol λ and tPol λ predominantly skipped over the AP site to generate a -1 frameshift mutation opposite the damaged site in almost every sequence context examined (Fig. 3). However, when replicating the 13-16/41-mer-AP-5'T substrate (Supplementary Table S2) that is most susceptible to strand slippage due to the presence of a template dT immediately 3' and 5' to the AP site (Fig. 3D), fPol λ preferentially generated a -2 frameshift mutation (72.3%) over generating a -1 frameshift mutation (24.1 %). This was the only sequence context in which fPol λ produced such a significant number of -2 frameshift mutations. Therefore, we hypothesize that fPol λ generated these single and double base deletions opposite the AP site through a dNTP-stabilized primer realignment mechanism and a homology-driven strand slippage mechanism, respectively (see Section 4). By using HT-SOSA analysis alone, we cannot say with certainty if fPol λ skipped the template dT at position -1 or position +1 during the generation of these -2 frameshift mutations (Fig. 3D). However, primer extension assays revealed that fPol λ readily incorporated a nucleotide opposite template position -1 in every sequence context examined by HT-SOSA, before pausing opposite the AP site (Fig. 2B and Supplementary Fig. S2). These findings strongly suggest that when replicating the 13-16/41-mer-AP-5'T damaged DNA substrate (Fig. 3D), fPol λ correctly incorporated dATP opposite template dT at position -1, prior to generating a -2 frameshift mutation. Taken together, these data are most consistent with the failure of fPol λ to incorporate a nucleotide opposite the template dT

at position +1 during the generation of these –2 frameshift mutations. Thus, these double base deletions were plotted opposite template positions 0 and +1 (Fig. 3D).

In contrast to fPol λ , dPol λ generated fewer double base deletions when template dT was positioned immediately 5' to the AP site (40.9%), but significantly more double base deletions when template dG (14.9%), dCdC (22.0%) or dCdT (14.8%) were 5' to the AP site (Fig. 3H, J, K and L). Therefore, in the absence of the BRCT domain, dPol λ generated fewer double base deletions when replicating the damaged DNA substrate 13-16/41-mer-AP-5'T (Fig. 3J), which is predicted to be the most prone to strand slippage, but yielded significantly more –2 frameshift mutations in almost every other sequence context tested. We concluded that the BRCT domain of human Pol λ possesses two sequence-dependent activities during TLS of an AP site: (i) promoting the bypass of AP sites through strand slippage when the AP site resides within a sequence context suitable for homology-mediated primer realignment; and (ii) inhibiting the non-specific generation of –2 frameshift mutations within sequence contexts that are not prone to strand slippage.

Like fPol λ , tPol λ only generated a significant number of –2 frameshift mutations when replicating the damaged 13-16/41-mer-AP-5'T substrate (Fig. 3M-R). However, the frequency of –2 frameshift mutations generated by tPol λ with this substrate was reduced to only 23.4% (Fig. 3P). Therefore, without the N-terminal BRCT and proline-rich domains, tPol λ retained the ability to facilitate a homology-driven strand slippage mechanism (Fig. 7C) of AP site bypass, but at a frequency 3.1- and 1.7-fold lower than fPol λ and dPol λ , respectively. These results indicate that the N-terminal BRCT and proline-rich domains of fPol λ act cooperatively promote a strand slippage mechanism of AP site bypass on templates prone to homology-driven primer realignment. In contrast to dPol λ , tPol λ produced very few –2 frameshift mutations when template dG or dC was positioned immediately 5' to the AP site (Fig. 3N, Q and R). Thus, after removal of the proline-rich domain from dPol λ , tPol λ ceased to produce double base deletions when replicating AP site-containing templates that are not susceptible to strand slippage. We concluded that the proline rich domain of Pol λ possesses an activity that stimulates the non-specific generation of –2 frameshift mutations during AP site bypass.

Pol β also preferentially skipped the AP lesion site to predominantly generate –1 frameshift mutations (Fig. 3T-X). However, when the base immediately 5' to the AP site was a template pyrimidine, Pol β generated a double-base deletion at a frequency of 19% to 28% (Fig. 3V, W and X). This result is inconsistent with the homology-driven strand slippage mechanism of AP site bypass as proposed for Pol λ . Instead, we hypothesize that during AP bypass, Pol β is capable of looping out both the AP site and the 5' template base when the template base is a pyrimidine (Fig. 7D). Remarkably, even though tPol λ and Pol β have the same domain structure (Fig. 1), tPol λ did not generate double base deletions when a template dC was immediately 5' to the AP site (Fig. 3Q and R). Thus, there are differences between the core dRPase and polymerase domains of tPol λ and Pol β that cause these enzymes to utilize alternate mechanisms of AP site bypass.

Consistent with the A-rule, when dPol λ , tPol λ or Pol β incorporated a nucleotide opposite the AP site, these polymerases preferentially incorporated dATP opposite the AP site in almost

every sequence context (Supplementary Fig. S3). In contrast, fPol λ preferentially incorporated dGTP opposite the AP site in most sequence contexts. Thus, the BRCT domain of fPol λ influenced the selection of an incoming dNTP opposite the AP site, potentially by influencing the architecture of the fPol λ active site during the polymerase catalytic cycle.

To determine if the ability of fPol λ , dPol λ , tPol λ and Pol β to incorporate a nucleotide opposite the AP site is dependent upon the gap size of the damaged DNA substrate, we also performed HT-SOSA with a damaged 16-19/41-mer-AP substrate that possessed a single-nucleotide gap (Supplementary Table S2). However, even after 1 h of extension, none of the polymerases tested were able to incorporate a nucleotide opposite the AP site within greater than 10% of TLS products based on the deletion error rate in Supplementary Tables S4-S7. Thus, we concluded that the failure of these polymerases to efficiently incorporate nucleotides opposite an AP site is not dependent upon the gap size of the DNA substrate.

3.3. The N-terminal domains of Pol λ influence polymerase fidelity opposite 8-oxodG lesions

To investigate if the N-terminal domains of Pol λ influence the efficiency of lesion bypass or polymerase fidelity during TLS of other oxidative lesions, we compared 8-oxodG bypass catalyzed individually by fPol λ , dPol λ , tPol λ , and Pol β . Primer extension assays utilizing DNA substrates that were either undamaged or contained a site-specifically placed 8-oxodG site (Table 1) demonstrated that the 8-oxodG lesion did not induce any noticeable pausing of these polymerases opposite the damaged site (Fig. 4). Thus, the N-terminal domains of human Pol λ do not influence the efficiency of 8-oxodG bypass.

To evaluate how the N-terminal domains of Pol λ influence polymerase fidelity during 8-oxodG bypass, we performed HT-SOSA analysis with fPol λ , dPol λ , tPol λ and Pol β by using undamaged and 8-oxodG-containing DNA substrates bearing a 7-nucleotide gap (Supplementary Table S2). Opposite 8-oxodG, we found that fPol λ was the most error-prone polymerase tested (Fig. 5), by predominantly misincorporating dATP (55.6%) or generating a single base deletion mutation (20.1%) over correctly incorporating dCTP (22.7%) (Fig. 6). Both dPol λ and tPol λ also predominantly misincorporated dATP opposite 8-oxodG at a ratio of 63.0% and 69.1%, respectively. However, dPol λ generated relatively few deletion mutations (3.2%) opposite the lesion site than fPol λ , and tPol λ produced fewer deletion mutations (1.1%) than either fPol λ or dPol λ opposite 8-oxodG (Fig. 5D and F). Thus, the N-terminal BRCT and proline-rich domains of Pol λ stimulated the generation of -1 frameshift mutations opposite the 8-oxodG lesion site during replication of the damaged 7-nucleotide gapped DNA substrate. Importantly, the overall rate of nucleotide incorporation catalyzed by fPol λ was nearly identical on the control and 8-oxodG-containing DNA substrates (Fig. 4A and B). Therefore, the ability of fPol λ to loop out a damaged site is not limited to situations where the polymerase is stalled, such as during the bypass of an AP site (Fig. 2B). HT-SOSA analysis also revealed that Pol β was the most accurate polymerase tested opposite 8-oxodG, by correctly incorporating dCTP (57.5%) opposite the lesion over misincorporating of dATP (38.8%) or generating a deletion mutation (3.4%). Thus, even though Pol β and tPol λ possess similar domain structure (Fig. 1), Pol β was 1.9-fold more

accurate than tPol λ for nucleotide incorporations opposite 8-oxodG when replicating the 7-nucleotide gapped damaged substrates (Fig. 5F and H).

Previously, we demonstrated that the fidelities of fPol λ , dPol λ , tPol λ and Pol β opposite undamaged template bases are dependent upon the gap size of the DNA substrate [23]. Therefore, we hypothesized that the actions of these polymerases opposite 8-oxodG would be altered when replicating DNA substrates containing a smaller gap. To test this prediction, we performed HT-SOSA analysis with DNA substrates that contained a single-nucleotide gap (Supplementary Table S2) and were either undamaged (16-18/40-mer-ctl) or possessed a site-specifically placed 8-oxodG lesion (16-18/40-mer-8oxodG). Opposite 8-oxodG within the single-nucleotide gapped 16-18/40-mer-8oxodG substrate, fPol λ still preferentially misincorporated dATP (56.0%) over correctly incorporated dCTP (43.2%) (Fig. 6B). However, the frequency of deletion mutations generated by fPol λ decreased by 33.5-fold to only 0.6%, with a concurrent increase in correct dCTP incorporation. Thus, the ability of fPol λ to generate deletion mutations opposite 8-oxodG was almost completely eliminated when replicating the single-nucleotide gapped DNA substrate, leading to a 1.9-fold increase in correct dCTP incorporation opposite 8-oxodG when replicating the damaged substrate containing a single-nucleotide gap compared to replication of the damaged substrate containing a 7-nucleotide gap (Fig. 6B). Remarkably, dPol λ and tPol λ correctly incorporated dCTP (74.0% and 52.1%, respectively) over misincorporating dATP opposite 8-oxodG within the single-nucleotide gapped 16-18/40-mer-8oxodG DNA substrate (Fig. 6B). Thus, similar to fPol λ , the frequency at which dPol λ and tPol λ correctly incorporated dCTP opposite 8-oxodG increased by 2.2-fold and 1.8-fold, respectively, when replicating the single-nucleotide gapped DNA substrate compared to the 7-nucleotide gapped substrate. The relative frequencies that fPol λ and Pol β incorporated dATP and dCTP opposite 8-oxodG within the single-nucleotide gapped DNA substrates as determined by HT-SOSA agree quite well with the frequencies determined by using pre-steady-state kinetic analysis with similar DNA substrates [27]. In contrast to fPol λ , dPol λ and tPol λ , the actions of Pol β opposite 8-oxodG were virtually unchanged by varying the gap size of the DNA substrate (Fig. 6B). We concluded that the fidelity of fPol λ and the Pol λ deletion mutations opposite 8-oxodG is highly dependent upon gap size, whereas the fidelity of Pol β is not.

4. Discussion

Human Pol λ and Pol β share 32% amino acid sequence identity within their conserved dRPase and polymerase domains [20]. However, Pol λ possesses two additional, non-enzymatic domains at its N terminus (Fig. 1). These additional domains likely contribute to the unique biological functions of Pol λ in vivo. Here, we sought to identify the contribution of the N-terminal domains of Pol λ toward the efficiency of lesion bypass and fidelity of nucleotide incorporation during the bypass of two of the most common forms of oxidative DNA damage, AP sites and 8-oxodG lesions. Our primer extension and HT-SOSA data show that (1) the BRCT domain increase the efficiency of AP bypass on gapped DNA substrates; (2) the BRCT and proline-rich domains cooperatively promote a homology-driven primer realignment mechanism of AP site bypass in certain sequence contexts; (3) the proline-rich domain non-specifically increases the generation of -2 frameshift mutations opposite an AP site while the BRCT domain negatively regulates this activity; and (4) both

N-terminal domains increase the frequency of frameshift mutations generated opposite 8-oxodG, suggesting that lesion looping-out is a general mechanism by which fPol λ bypasses damaged sites, such as AP and 8-oxodG lesions. Overall, these primer extension and HT-SOSA indicate that the N-terminal domains of Pol λ increase the ability of Pol λ to extend primer/template pairs with limited homology. This role of the N-terminal domains may be important for the proposed function of Pol λ within the NHEJ pathway, especially in situations where damaged nucleotides must be bypassed during gap-filling DNA synthesis.

Previous studies of AP bypass by fPol λ and Pol β suggested that fPol λ primarily generates frameshift mutations opposite the abasic site whereas Pol β can either produce a frameshift or incorporate dATP [32]. Our HT-SOSA data indicate that fPol λ , dPol λ , tPol λ , and Pol β predominantly skip the AP site to generate -1 or -2 frameshift mutations, which are likely produced by different mechanisms of AP site bypass. DNA polymerases have been proposed to generate frameshift mutations through three separate mechanisms (Fig. 7) (reviewed in reference [36]), namely dNTP-stabilized realignment [33], direct misincorporation realignment [37,38], and the classical Streisinger model of homology-driven strand slippage [39]. Our data are most consistent with fPol λ bypassing the AP site by using a dNTP-stabilized realignment mechanism to generate -1 frameshift mutations (Fig. 7A), or a homology-driven strand slippage mechanism to generate -2 frameshift mutations (Fig. 7C). Consistently, such a homology-driven primer realignment mechanism for Pol λ has also been predicted based upon kinetic studies of AP bypass by Pol λ [32]. Interestingly, dPol λ generated -2 frameshift mutations in multiple sequence contexts while Pol β did so when the template base immediately 5' to the AP site was a pyrimidine (Fig. 3). Because these sequences are not predicted to be susceptible to homology-driven strand slippage, we propose that these -2 frameshift mutations were derived by dNTP-stabilized realignment, wherein both the AP site and the base 5' to the AP site are looped out (Fig. 7D). Overall, these findings indicate that even though fPol λ and Pol β possess similar polymerase core domains, these two X-family enzymes use alternative mechanisms of AP site bypass. Furthermore, these findings indicate that the ability of Pol λ to promote homology-driven primer realignment is possessed by the dRPase and polymerase core domains of Pol λ (Fig. 1), although the N-terminal domains play a significant role in promoting this process.

The AP bypass efficiencies of the four enzymes tested were qualitatively determined to be fPol λ > dPol λ = tPol λ = Pol β (Table 2) based upon the calculated t_{50}^{bypass} values with the 18-16/41-mer-AP-5'G DNA substrate (Table 1). Interestingly, we found that both fPol λ and dPol λ selectively generated -1 frameshift mutations opposite the AP site when replicating a similar 13-16/41-mer-AP-5'G DNA substrate (Fig. 3B and H), presumably through a dNTP-stabilized primer realignment mechanism. Thus, the N-terminal domains of fPol λ appear to increase the efficiency at which fPol λ bypassed the AP site by using the dNTP-stabilized primer realignment mechanism (Fig. 6A), in addition to promoting the frequency by which fPol λ utilized the homology-driven strand slippage mechanism (Fig. 6C).

We found that the most common mutation induced by 8-oxodG bypass catalyzed by fPol λ , dPol λ , tPol λ , and Pol β was dATP misincorporation opposite 8-oxodG (Fig. 6B). Interestingly, the gap size strongly influenced the actions of fPol λ , dPol λ and tPol λ opposite 8-oxodG (Figs. 5 and 6). The frequency at which fPol λ , dPol λ and tPol λ correctly

incorporated dCTP opposite 8-oxodG was increased approximately 2-fold when replicating DNA substrates containing a single-nucleotide gap compared to a 7-nucleotide gap. Importantly, the miscoding potential of 8-oxodG is strongly influenced by the ratio of the *anti* and *syn* conformations of 8-oxodG within a polymerase active site. Thus, this finding suggests that the gap size influenced the preference of 8-oxodG to adopt the *anti* or *syn* conformation within the active site of fPol λ , dPol λ and tPol λ , indicating that the gap size of a bound DNA substrate induces significant differences in the active site architecture of Pol λ . In contrast, the actions of Pol β were nearly unchanged by altering the gap size (Fig. 6B).

In humans, 8-oxodG•dA mismatches are repaired through the BER pathway by the removal of the mismatched dA base through the action of MYH adenine glycosylase followed by a nucleotide incorporation opposite 8-oxodG by a gap-filling DNA polymerase. Because Pol β was less error prone than fPol λ opposite 8-oxodG when replicating both the single- and 7-nucleotide gapped DNA substrates (Fig. 6B), our data suggest that for the sake of genomic stability, Pol β is more suitable than fPol λ to perform this function during either short patch or long patch BER *in vivo*.

Crystallographic studies indicate that Pol β and tPol λ interact with both the 3'-hydroxyl of an upstream primer strand and the 5'-phosphate of a downstream primer strand of a single-nucleotide gapped DNA substrate through the residues of the polymerase active site and the dRPase domain, respectively [34,35]. Remarkably, tPol λ can remain in contact with both the aforementioned 3'-hydroxyl and the 5'-phosphate groups when the gap size is increased to two by scrunching out the unpaired template base that is immediately 5' to the templating base into an extrahelical position within a binding pocket consisting of L227, H511 and R514 [40]. Furthermore, crystallographic studies demonstrate that when tPol λ generates a single-nucleotide deletion mutation through primer/template realignment, the 3' template nucleotide, which is two positions from the templating base, is looped out and this extrahelical nucleotide is stabilized through a hydrogen bonding interaction between the 5' phosphate adjacent to the flipped out base and the side chain of K544 [41]. Unfortunately, no crystal structures of fPol λ and dPol λ are available. Thus, it has been difficult to assign an exact role to the N-terminal domains of Pol λ during nucleotide binding and incorporation. Based on our HT-SOSA data and the abovementioned crystal structures [34,35,40,41], we propose a model wherein the N-terminal domains of Pol λ stabilize frameshift intermediates by maintaining one or more looped-out template nucleotides in extrahelical positions (Fig. 8). The looped-out nucleotide(s) will likely interact with the residues in both N-terminal domains of Pol λ . The models described in Fig. 8 are consistent with our findings that the N-terminal domains of Pol λ increase the efficiency of AP site bypass (Table 2), the frequency of -2 frameshift mutations produced by primer/template realignment during AP site bypass (Fig. 3), and the generation of -1 frameshift mutations opposite 8-oxodG (Fig. 5B).

In conclusion, our study supports a model wherein the N-terminal domains of Pol λ promote bypass of damaged sites through looping-out damaged bases. This activity may also allow Pol λ to loop out undamaged bases to stimulate the extension of primer/template junctions of limited homology, such as those encountered during the gap filling step of the NHEJ repair pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health grant (GM079403) to Z.S. We thank Jason D. Fowler for the purification of fPol λ , dPol λ , tPol λ and Pol β . In addition, we thank Dr. Pearly Yan and John Curfman at The Ohio State Nucleic Acid Shared Resource for their aid in performing the next-generation sequencing.

Abbreviations

8-oxodG	8-oxo-7,8-dihydro-2'-deoxyguanosine
AP	apurinic/aprimidinic
BER	base excision repair
BSA	bovine serum albumin
BRCT	BRCA1 C-terminal domain
dNTP	2'-deoxynucleoside 5'-triphosphate
dPolλ	deletion of human DNA polymerase λ (132-575)
dRPase	5'-2-deoxyribose-5-phosphate lyase domain
fPolλ	full-length human DNA polymerase λ (1-575)
HT-SOSA	high-throughput short oligonucleotide sequencing assay
NHEJ	non-homologous end joining
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
Polβ	human DNA polymerase β
tPolλ	truncated human DNA polymerase λ (245-575) lacking the N-terminal domains
TLS	translesion DNA synthesis

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.dnarep.2014.07.008>.

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Figure 1.

Schematic domain structures of human fPolλ, dPolλ, tPolλ and Polβ. The nuclear localization signal (NLS), BRCA1 C-terminal (BRCT) domain, proline-rich domain, 5'-2-deoxyribose-5-phosphate lyase (dRPase) domain, and polymerase core domain of each full-length or truncated enzyme are labeled, and the amino acid residue numbers are indicated below each diagram.

Figure 2.

AP site bypass individually by fPolλ, dPolλ, tPolλ and Polβ with gapped DNA substrates. A pre-incubated solution of 100 nM of an indicated polymerase and 100 nM of 5'-[³²P]-labeled 18-16/41-mer-ctl (A, C, E and G), or 18-16/41-mer-AP-5'G (B, D, F and H) was rapidly mixed with a solution of all four dNTPs (200 μM each). The reaction mixtures were first quenched at indicated times with 0.37 M EDTA and then resolved by using denaturing PAGE. The sizes of important products are indicated. The 22nd position is marked with an

asterisk (*) to denote the position of the AP site within the damaged template while the 25th position is immediately adjacent to the 5'-terminus of the downstream primer.

Figure 3.

The N-terminal domains of Polλ influence the generation of -1 or -2 frameshift mutations opposite or adjacent to an AP site in a sequence-dependent manner. Histograms of the relative number of insertion (crosshatched), deletion (black), and substitution (white) mutations generated by fPolλ (A through F), dPolλ (G through L), tPolλ (M through R), or Polβ (S through X) as a percentage of the total dNTP incorporations are shown at each template position. The identity and position of each template base relative to the position of the AP site “X” within the damaged templates are indicated.

Figure 4.

The N-terminal domains of human Polλ do not influence the efficiency of 8-oxodG bypass. A pre-incubated solution containing 100 nM of the indicated polymerase and 100 nM of 5'-[³²P]-labeled 18-15/40-mer-ctl (A, C, E and G) or 18-15/40-mer-8oxodG (B, D, F and H) was rapidly mixed with a solution of all four dNTPs (200 μM each). The reaction mixtures were quenched at the indicated times with 0.37 M EDTA and resolved by using denaturing PAGE. The sizes of important products are indicated. Specifically, the 22nd position is

marked with an asterisk (*) to denote the position of the 8-oxodG lesion site within the damaged template.

Figure 5.

The N-terminal domains of human Polλ increase the generation of -1 frameshift mutations opposite 8-oxodG. Histograms of the relative number of insertion (crosshatched), deletion (black), and substitution (white) mutations generated by fPolλ (A and B), dPolλ (C and D), tPolλ (E and F), or Polβ (G and H) as a percentage of the total dNTP incorporations are shown at each template position. The indicated template positions are relative to the 8-oxodG lesion site “Y” within the damaged template.

Figure 6.

Gap size influences the preferred action of fPolλ, dPolλ, tPolλ and PolB opposite 8-oxodG.

The relative numbers of nucleotide incorporations, deletion mutations, or insertion mutations produced by fPolλ, dPolλ, tPolλ, or Polβ opposite undamaged dG (A) or 8-oxodG (B) with DNA substrates containing a 7-nucleotide or single-nucleotide gap are indicated.

Figure 7.

Mechanisms by which Pol λ and Pol β generate frameshift mutations opposite a lesion site denoted as “X”. (A) dNTP-stabilized realignment. The lesion site is flipped into an extrahelical position and the resulting terminus is realigned by an incoming dNTP correctly base pairing with the next templating base. Continuous primer extension leads to a -1 frameshift mutation. (B) Incorporation-mediated realignment. After the incorporation of a dNTP opposite the lesion site, the lesion is flipped into an extrahelical position and the primer 3' terminus is realigned with the template base 5' to the lesion site. Further extension of the primer generates a -1 frameshift mutation. (C) Homology-driven strand slippage. Both the lesion and the adjacent 3' template base are looped out and the primer 3' terminus is realigned with a template base 5' to the lesion site. Extension generates a -2 frameshift mutation. (D) dNTP-stabilized realignment to generate a -2 frameshift. Both the lesion and the adjacent 3' template base are looped out, and the resulting realignment is stabilized by an

incoming dNTP correctly base pairing with a template base 5' to the lesion site. Continuous primer extension leads to a -2 frameshift mutation.

Figure 8.

Roles of the N-terminal domains of Polλ during frameshift generation. Models of -1 (A) or -2 (B) frameshift generation during Polλ-catalyzed TLS. An illustration of full-length Polλ is shown bound to a gapped DNA substrate containing a lesion site. Once Polλ encounters a template lesion site (red color), the lesion (and an adjacent base) will be looped out and interact with the BRCT and proline-rich domains. Extension of the frameshift intermediate leads to the generation of a -1 or -2 frameshift mutation.

Scheme 1.

Lesion bypass products were generated by the extension of a gapped control or damaged DNA substrate. The 5' phosphate of the downstream primer is represented as "P". The newly synthesized strands were isolated by denaturing PAGE and subsequently amplified by two rounds of PCR to add a four nucleotide barcode and the adapter sequences necessary for next-generation sequencing. The adapter sequences are shown as white bars. The barcode is indicated as 'BBBB'. The flanking region used to calculate the background error rates and

the sampled sequence used for lesion bypass analysis are indicated. The position of the AP site is depicted as “X”.

Table 1DNA substrates used for primer extension experiments^a

18/41-mer-ctl	5'-CGCAGCCGTCCAACCAAC 3'- GCGTCGGCAGGTTGGTTGAGT A GCAGCTAGGTTACGGCAGG-5'
18/41-mer-AP-5'G	5'-CGCAGCCGTCCAACCAAC 3'- GCGTCGGCAGGTTGGTTGAGT X GCAGCTAGGTTACGGCAGG-5'
18-16/41-mer-ctl	5'-CGCAGCCGTCCAACCAAC _p GATCCAATGCCGTCC-3' 3'- GCGTCGGCAGGTTGGTTGAGT A GCAGCTAGGTTACGGCAGG-5'
18-16/41-mer-AP-5'G	5'-CGCAGCCGTCCAACCAAC _p GATCCAATGCCGTCC-3' 3'- GCGTCGGCAGGTTGGTTGAGT X GCAGCTAGGTTACGGCAGG-5'
18-15/40-mer-ctl	5'-CGCAGCCGTCCAACCAAC _p GATCCAATGCCGTCC-3' 3'-GCGTCGGCAGGTTGGTTGAGT G CAGCTAGGTTACGGCAGG-5'
18-15/40-mer-8oxodG	5'-CGCAGCCGTCCAACCAAC _p GATCCAATGCCGTCC-3' 3'-GCGTCGGCAGGTTGGTTGAGT Y CAGCTAGGTTACGGCAGG-5'

^a**X** represents the AP lesion site while **Y** represents the 8-oxodG lesion site. The subscript 'p' indicates that the 5'-end of the downstream primer is phosphorylated.

Table 2

AP bypass efficiencies on gapped and non-gapped DNA substrates.

Enzyme	Gapped DNA substrates ^a			Non-gapped DNA substrates ^b		
	t_{50}^{bypass} (s) ^c	t_{50} (s) ^d	t_{50}^{bypass}/t_{50}	t_{50}^{bypass} (s) ^c	t_{50} (s) ^d	t_{50}^{bypass}/t_{50}
hPol λ	290	8.7	33	>10,800	340	>32
dPol λ	470	3.8	123	>10,800	230	>47
tPol λ	490	4.0	122	>10,800	11	>980
Pol β	470	0.94	500	430	1.8	240

^aControl 18-16/41-mer-ctl and damaged 18-16/41-mer-AP-5′G substrates.

^bControl 18/41-mer-ctl and damaged 18/41-mer-AP-5′G substrates.

^cCalculated as the time required to bypass 50% of the AP sites at position 22 in Fig. 2.

^dCalculated as the time required to bypass 50% of the corresponding dA sites at position 22 in Fig. 2.